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Influence of Turbidity on Photometric Assays: A Blank Sample Must Always Be Used

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Summary: The authors studied the influence of sample turbidity on photometric assays at different wavelengths. Turbidity was created by using a mixture of hydrazine sulfate and hexamethylene tetramine, and a dye solution (*p*-nitrophenol, cobalt nitrate).

This work demonstrates the necessity of using a blank sample, for example in the glucose assay (glucose oxidase/peroxidase). The influence of turbidity on an assay depends upon the instrument. Knowledge of this influence is essential for the evaluation of the viablity of an assay in hyperlipemic serum.

Einfluß der Trübung auf photometrische Bestimmungen: Muß jeweils ein Proben-Leerwert benutzt werden?

Zusammenfassung: Die Autoren untersuchten den Einfluß der Trübung von Proben auf photometrische Bestimmungen bei verschiedenen Wellenlängen. Die Trübung wurde bewirkt mit Hilfe einer Mischung von Hydrazinsulfat und Hexamethylentetramin und einer Farblösung (*p*-Nitrophenol, Cobaltnitrat).

Diese Arbeit berichtet über die Notwendigkeit, einen Probenleerwert zu benutzen, z. B. bei der Glucosebestimmung (Glucoseoxidase/Peroxidase-Methode). Der Einfluß der Trübung auf eine Bestimmung ist bei den verschiedenen Instrumenten unterschiedlich. Die Kenntnis dieses Einflusses ist unentbehrlich für die Bewertung der Durchführbarkeit einer Bestimmung bei hyperlipämischem Serum.

Introduction

With the development of direct methods (without deproteinisation) (1–2) it is necessary to emphasize the importance of the "blank sample" (i.e. the sample containing plasma and buffer) particularly for lipemic sera or plasmas. Does turbidity affect the result of an assay? Is the use of a blank sample indispensable, and is it sufficient to correct for this effect? These questions are examined here, using the wavelengths normally used in the laboratory.

Figure 1 shows the frequencies of various turbidity assay values found for presumably healthy individuals undergoing medical checkups at the "Centre de Médecine Préventive" in Nancy.

Fifty per cent of the population of the individuals had not fasted; 2.5 % of those who had fasted had turbidities greater than 0.4 absorbance units. Eleven per cent of the population that had not fasted had high turbidities, i.e. greater than 0.7 absorbance units. The problem presented by the consequences and the frequency of turbidity should be considered for any new method and for any instrument (6,9).

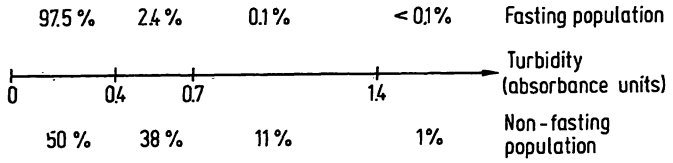

Fig. 1. Turbidity distribution of the plasma samples.

Materials and Methods

Turbidity was determined with a GSA II Greiner (3) by diluting 20 µl of the sample in 400 µl of NaCl and NaF solution. The respective concentration of these reagents in the final volume was 0.118 and 0.02 mol/l.

Zero was established with NaCl solution, using 578 nm. When the wavelength is increased (i.e. to avoid interference by bilirubin and hemoglobin), the light transmission of a turbid solution is decreased. The results are expressed in absorbance units.

The tests were performed by adding a turbid reference solution to a dye solution of known absorbance, and measuring the effect at a given wavelength.

Apparatus

The Aminco DW_2 spectrophotometer (fig. 2b) is a high performance tungsten lamp instrument. It facilitates the absorbance

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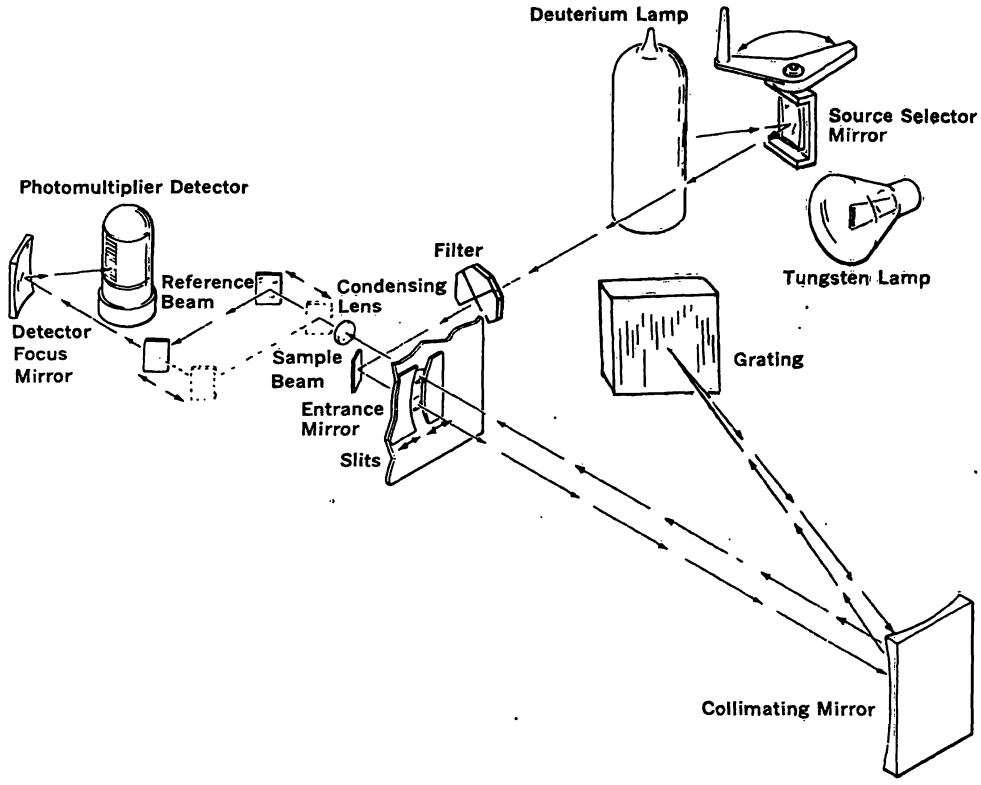

Fig. 2a

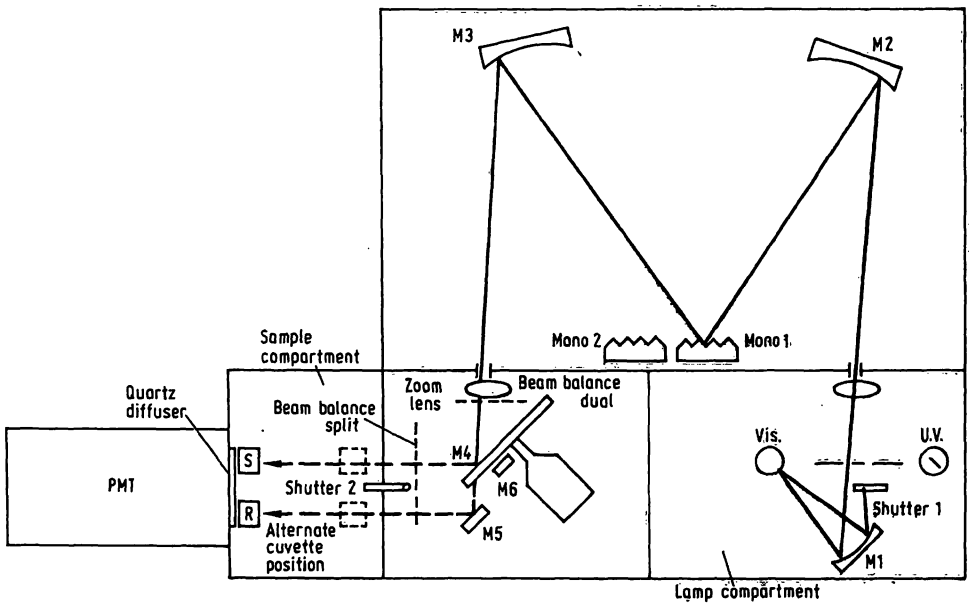

Fig. 2b

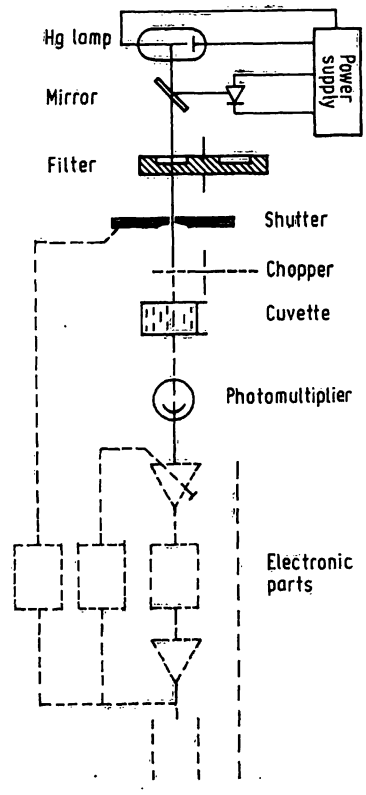

Fig. 2c

Fig. 2. Optical arrangements of the spectrophotometers used.
Fig. 2a. Beckman DB GT Fig. 2b. Aminco DW_2: PMT (photomultiplier) Fig. 2c. Greiner GSA II

reading in a turbid medium by permitting the photomultiplier to be positioned 0.5 cm from the cuvette. This spectrophotometer served as the control for all the tests carried out in this work, and it was used to plot the absorbance spectrum as a function of wavelength for each of the various mixtures used. Figure 3 shows an example of two spectra. The control curve is drawn to assure that the addition of the turbid solution does not provoke a displacement of the absorption peak of the coloured solution.

Two other instruments used: the Greiner GSA II photometer (fig. 2c) with an interference filter and a mercury vapor lamp from an automatic device; and the Beckman DB GT spectrophotometer (fig. 2a) which has a hydrogen lamp and a tungsten lamp, and wavelength selection by a diffraction grating[1]).

Experimental method

Reagents

For the studies at 366 nm, potassium dichromate dye solution (340 µmol/l in 1 mmol/l perchloric acid) was used (7–9–14).

For the studies at 405 nm, *p*-nitrophenol (60 µmol/l) in an alkaline solution (20 mmol/l) was used.

For the studies at 436–492–546nm, cobalt nitrate (680 mmol/l) in 100 g/kg HNO_3 was used for the three wavelengths.

For the studies at 578 nm, an iron-ferrozine mixture (70 µmol/l of ferrous iron in 6.8 µmol/l ferrozine solution) was used.

All these reagents were used undiluted, or diluted 1/2, 1/3, or 1/4 in bidistilled water, in order to achieve an appropriate range of absorbance: 1.6, 0.8, 0.4, 0.2 absorbance units (See an example tab. 1). For the turbid solution equal volumes of hydrazine sulfate (70 mmol/l) and hexamethylene tetramine (0.7 mol/l) were mixed at the ambient temperature and allowed to stand for 24 hours (8).

The suspension is stable for one month. The original solution is used undiluted, or diluted (1/4, 1/8, 1/16); as appropriate. Using the Greiner, these solutions gave turbidity values of 5.3, 1.4, 0.7 0.3 absorbance units. The dilutions were chosen to give values in the same range as those of the patients' samples. N.B. It is necessary during the assay to keep the solution continually agitated.

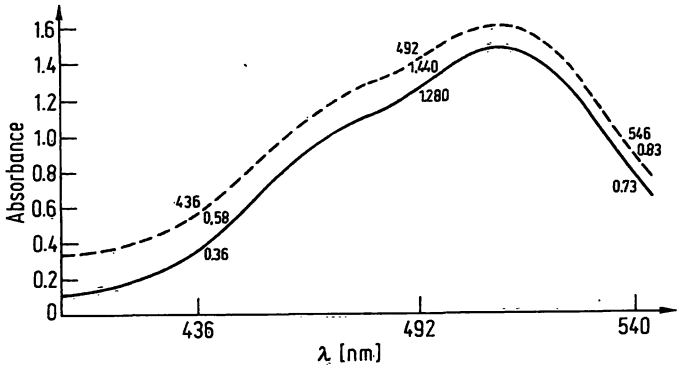

Fig. 3. Spectra are traced on the Aminco DW_2 spectrophotometer.
– – – dye solution + initial turbid solution
—— dye solution + H_2O

[1]) Beckman DB GT Instruments France, Chemin des Bourdons, F-93220 Gagny,
DW_2 Aminco Kontron, 6 rue des Frères Caudron, F-78140 Velizy-Villacoublay,
GSA II Greiner, Gaswerkstraße, CH-4900 Langenthal, Schweiz

Tab. 1. An example at 366 nm of experimental protocol

λ_{nm}	Dye solution (400 µl) + Turbid solution (20 µl)
366	initial dye: turbid solution (pure); diluted (1/4); diluted (1/8); diluted (1/16)
	diluted 1/2
	diluted 1/3
	diluted 1/4

$K_2Cr_2O_7$

Analysis

Four tests A_1, A_2, A_3 and A_4 are represented in table 2.

- A_1 corresponds to the measurement against water of the absorbance of the turbid solution (turbidity assay made on the GSA II).
- A_2 corresponds to the measurement against water of the dye solution alone (this was the reference absorbance due to the dye).
- A_3 compares the absorbance of the combined dye and turbid solutions with that of water (simulating a measurement for which a blank sample is not used)
- A_4 compares the absorbance of the combined dye and turbid solutions with that of the turbid solution (simulating a measurement for which a blank sample is used)

The influence of the turbidity was excluded by taking the difference between the absorbance obtained in A_3 and A_2; and the possible necessity of a blank sample was determined by taking the difference between A_4 and A_2. For the chosen wavelength, we used the appropriate dye solution at different dilutions as mentioned earlier. For each dilution we studied the influence of the addition of four turbid solutions, keeping the ratio $\frac{\text{turbid solution}}{\text{isotonic solution}}$ equal to the ratios $\frac{\text{sample}}{\text{isotonic solution}}$ used in the turbidity measurement.

Tab. 2. Procedure method.

turbid solution
dye solution

Measured absorbance	Test absorbance	Control absorbance
A_1	Turbid solution	Water
A_2	Dye solution	Water
A_3	Dye solution + turbid solution	Water
A_4	Dye solution + turbid solution	Turbid solution

$A_3 - A_2$ expresses the difference in absorbance due to the turbidity

$A_4 - A_2$ reflects the influence of the blank sample

Results

Relationship between turbidity and concentration of the solutions

In studying whether there was a relationship between the concentration of the solutions and the magnitude of the interfering phenomenon (in this case the turbidity) we used the main absorbance region of the dye solutions. In figure 4, the influence of the turbidity, A_3-A_2 , is plotted against the absorbances of the dye solutions, A_2.

At 366 nm and perhaps at 405 nm a relationship appeared to exist between the turbidity and the concentration of the dye solution; at the other wavelengths, the influence of the turbidity was not linked to the value of the absorbance.

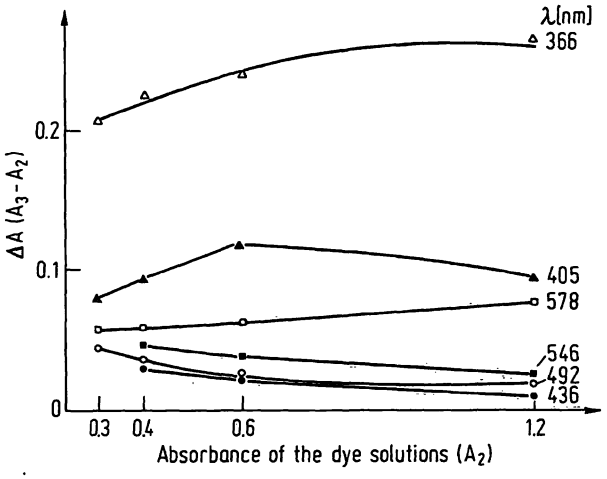

Fig. 4. GSA II – Effect of turbidity on absorbance (Turbidity = 1.4) as a function of the concentration of the solution.

Difference in absorbance, A_3-A_2, due to turbidity, as a function of wavelength

These tests were carried out in order to quantify the observed interference and to determine whether the blank sample was indispensable. It is certain that the influence of the light scattered during the course of a photometric reading is very great (3). What is measured is an apparent absorbance. One important factor is the optics of the apparatus. For this reason, we have shown the results obtained on each instrument (4,5,6,10) separately.

The difference in absorbance, A_3-A_2, due to the addition of the turbidity, is indicated on the vertical axis in figures 5, 6, and 7. It is shown as a function of the various wavelengths for the entire range of turbid solutions.

The results obtained on the Aminco DW_2 spectrophotometer (figure 5) are in agreement with those of other authors (3). They show that the absorbance due to the turbidity, A_3-A_2, decreased with increasing wavelength. The higher turbidity, the greater is the difference found, in particular at 366 nm, where an A_3-A_2 value of 0.5

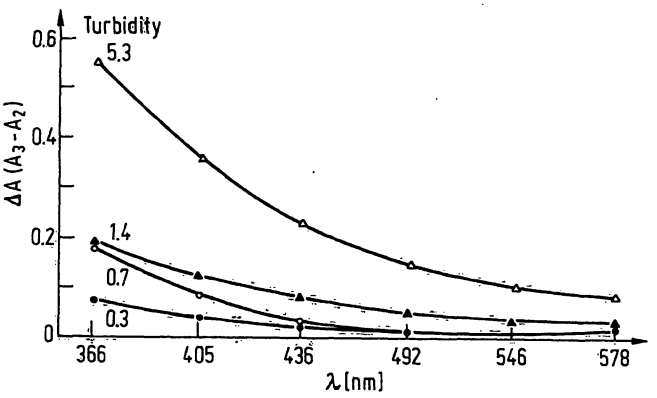

Fig. 5. Aminco DW_2 – Effect of turbidity on absorbance. On the vertical axis is shown the difference in absorbance, A_3-A_2, brought about by the turbidity, as a function of wavelength.

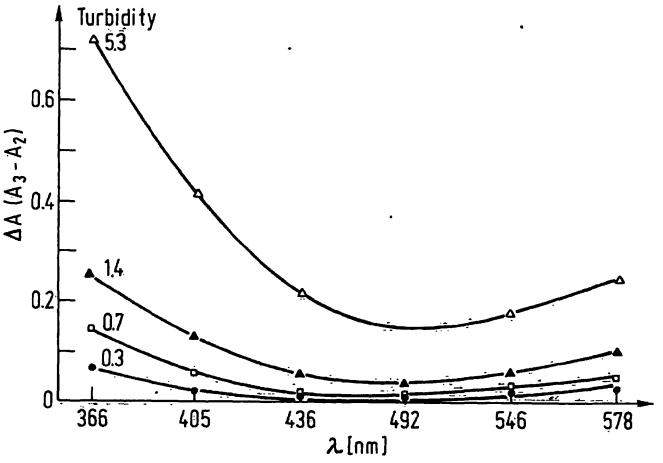

Fig. 6. Greiner GSA II – Effect of turbidity on absorbance. On the vertical axis is shown the difference in absorbance, A_3-A_2, brought about by the turbidity, as a function of wavelength.

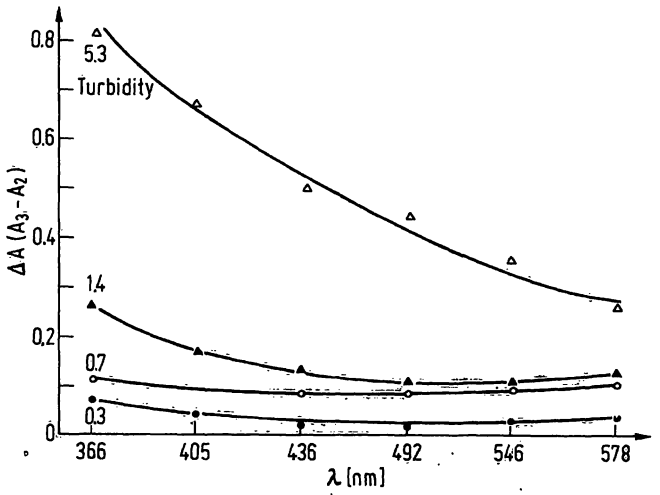

Fig. 7. Beckman DBGT – Effect of turbidity on absorbance. On the vertical axis is shown the difference in absorbance, A_3-A_2, brought about by the turbidity, as a function of wavelength.

absorbance units was found for a turbidity of 5.3. The value at this wavelength is therefore too high, and it represents an error in specificity.

The values observed with the GSA II are presented in the same manner (fig. 6). Over the range of wavelengths, the results were comparable to those obtained with the Aminco DW_2, except at 366 nm, where the influence of turbidity was still greater for the GSA II. It should be noted that at the wavelengths higher than 492 nm, the difference A_3-A_2 no longer decreased, but increased. This phenomenon might be explainable in terms of two physical laws with opposite effects, that are brought into play during measurement in a turbid medium.

First, the *Rayleigh* law (11) affirms that the increase in absorbance with turbidity is inversely proportional to the fourth power of the wavelength. This is verified up to 492 nm.

$$I_D = KI_o \frac{n^2-n_o{}^2}{n^2+2n_o{}^2} \frac{-NV^2}{\lambda_0{}^4}$$

I_D: intensity of scattered radiation
I_0 : intensity of incident radiation
V : volume of particles
N : number
K : constant
n_o: index of refraction in medium
n : index of refraction of the particles

Secondly, it is known that the light transmitted in a turbid medium decreases with increasing wavelength (3, 12, 13). The measured absorbance thus increases. Above 492 nm, this phenomenon becomes preponderant and may explain the rise in this curve (fig. 6) between 492 and 578 nm. Such a rise was not noted for the Aminco DW_2 instrument (fig. 5), perhaps because the position of the photomultiplier was specially designed for turbid solutions.

The values on the vertical axis, for the Beckman DB GT device (fig. 7), are higher than for the other two photometers, irrespective of the wavelength and the turbidity used. The second law, concerning transmitted light, may be of importance for this apparatus from wavelength 405 nm onwards.

Is the use of a blank sample necessary?

The difference in absorbance A_4-A_2, which reflects the utility and the effectiveness of a blank sample, should be zero or close to it; this indicates that the influence of the turbidity has been effectively eliminated by the use of the blank. A general conclusion as to the effectiveness of using a blank sample cannot be established because each photometer or spectrophotometer has its own optical characteristics. The use of a blank sample for the tests on the Aminco (fig. 8) was ineffective: at 366 nm, the residual absorbance, A_4-A_2, was 25 % of the calculated, A_3-A_2.

The GSA II photometer behaved similarly; the residual absorbance reappeared weakly at 578 nm (fig. 9).

The residual absorbance, A_4-A_2 found with the Beckman DB GT spectrophotometer (fig. 10) was higher than for the other instruments: it was on average 0.05 absorbance unit. It seems that this instrument does not eliminate the effect of turbidity below a threshold peculiar to itself.

It appears that all the instruments studied require the use of a blank sample at all wavelengths, especially for highly turbid samples. As it is difficult or impossible to select samples according to their level of turbidity before assaying them, it follows that the blank sample should be used systematically.

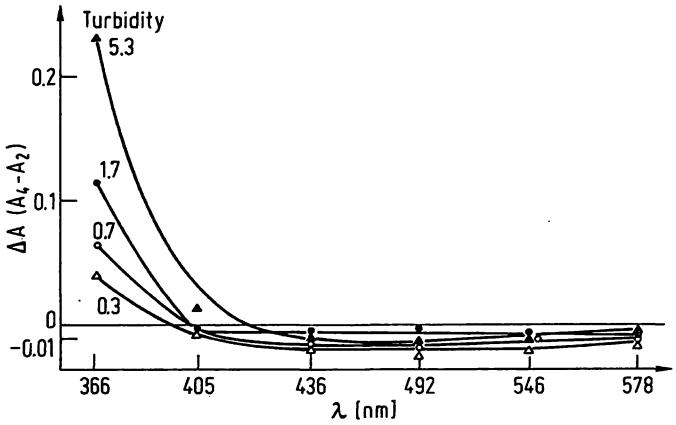

Fig. 8. Aminco DW_2 – Use of the blank sample.
On the vertical axis is shown the difference in absorbance, A_4-A_2, which represents the residual absorbance after elimination of that of the blank sample, as a function of wavelength.

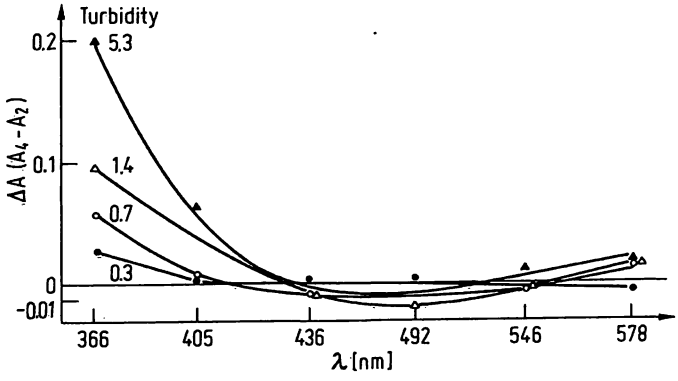

Fig. 9. Greiner GSA II – Use of the blank sample.
On the vertical axis is shown the difference in absorbance, A_4-A_2, which represents the residual absorbance after elimination of that of the blank sample, as a function of wavelength.

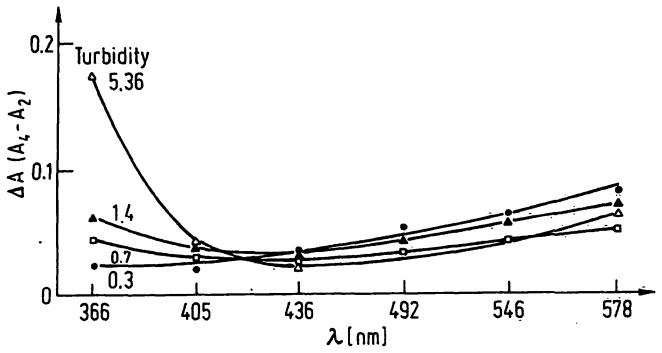

Fig. 10. Beckman DB GT – Use of the blank sample.
On the vertical axis is shown the absorbance difference, A_4-A_2, which represents the residual absorbance after elimination of that of the blank sample, as a function of wavelength.

Applications

A study was made of the usefulness of a blank sample for assaying glucose by the glucose oxidase/peroxidase (GOD/POD) method.

The tests were made on about 100 presumably healthy patients who came to the Centre de Médecine Préventive for a medical checkup.

The plasma glucose of each individual was assayed four times:

- on the Greiner GSA II automatic spectrometer;
- by the glucose hexokinase method;
- by the glucose oxidase/peroxidase (GOD/POD) method;
- and manually, following the procedures of the automatic GOD/POD method. The tests were made with and without a blank sample.

The absorbance values of the blank samples are noted in table 3a. The reading was at 546 nm.

Tab. 3a. Absorbance values of the blank samples.
n = number S = Standard Deviation

GOD POD manual method	n population P_1	Average	S
Blank sample absorbance	100	0.004 dA (or 0.128 mmol/l of glucose)	0.002

Results

The population of plasma samples was divided according to turbidity:

Population P_1 < turbidity 0.6 ≤ Population P_2

100 patients About 11 patients

For population P_1, the average absorbance of the blank samples (containing plasma and buffer) was 0.004 absorbance units ±0.002; a high dilution ratio, I/125, was used.

0.004 absorbance units expressed as a glucose concentration represents 0.128 mmol/l (table 3a). This value was found for population P_1, and the average of the measurements carried out manually was compared with and without a blank sample (tab. 3b). The average of the differences was 0.134 mmol/l. The method without a blank sample presents a positive interference due to the turbidity of the samples.

Population P_2 was about 10 % of the patients. Each case is presented individually. There was good correlation between the glucose hexokinase and the glucose oxidase tests in the manual method with a blank (tab. 4a). On the other hand, if the GOD/POD without a blank is compared with the two methods using a blank, there was a systematic average shift of 0.478 mmol/l. Patient samples of very high turbidity are relatively rare at the Centre de Médecine Préventive (tab. 4b), but such samples are much more common in the hospital environment. The difference noted between the methods with and without a blank corresponds to the modification of absorbance brought about by the turbidity of the sample itself.

Conclusion

For each type of instrument it appears necessary to determine, as a method is evaluated or improved, the influence of the turbidity of the sample and the accuracy of the assay. It must also be verified that no reagent modifies this turbidity, either diminishing it, as does EDTA, or increasing it. Examining the results of the analysis of the correlation between certain parameters could lead to some erroneous interpretations. The influence of the turbidity of a plasma sample is such that it is necessary to utilize a blank sample. Our results apply only to the spectrophotometers studied here. It is certain that the problem must be taken into account for each type of apparatus.

Tab. 3b. Study of the importance of the blank sample. Application to the assay of glucose for Population P_1 (P_1 have turbidity < 0.6).
n = number (s.) = significant S = Standard Deviation

GOD POD manual method	n population P_1	Average mmol/l	Average of the differences	S	t
Measurement without blank sample	92	5.78	0.134	0.07	18.94 (s.)
Measurement with blank sample	92	5.65			

Tab. 4a. Study of the importance of turbidity on the results of glucose assay for Population P_2 (P_2 have turbidity ⩾ 0.6).

Patient No. (P_2)	Turbidity of plasma sample absorbance units	Hexokinase (mmol/l)	GOD/PAP with blank (mmol/l)	GOD/PAP without blank (mmol/l)
200 990	0.97	5.27	5.26	5.73
201 006	1.37	5.15	5.13	5.68
201 008	0.80	5.80	5.81	6.15
201 011	1.07	4.39	4.66	5.09
201 029	0.62	5.10	5.18	5.53
201 033	0.71	5.44	5.54	5.89
201 039	0.90	4.65	4.87	5.27
201 125	1.15	5.86	5.68	6.14

Tab. 4b. Special cases: test on the three methods on the GSA II. The blank sample absorbance was evaluated manually.

Patient No. (P_2)	Turbidity of plasma sample absorbance units	Absorbance of blank sample	Hexokinase (mmol/l)	GOD/PAP with blank (mmol/l)	GOD/PAP without blank (mmol/l)
201 600	3.12	0.066 = 2.1 mmol/l	5.27	5.74	7.55
201 603	21.00	0.117 = 3.74 mmol/l	5.33	5.80	9.63
201 637	0.92	0.011 = 0.3 mmol/l	6.05	6.08	6.24

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